



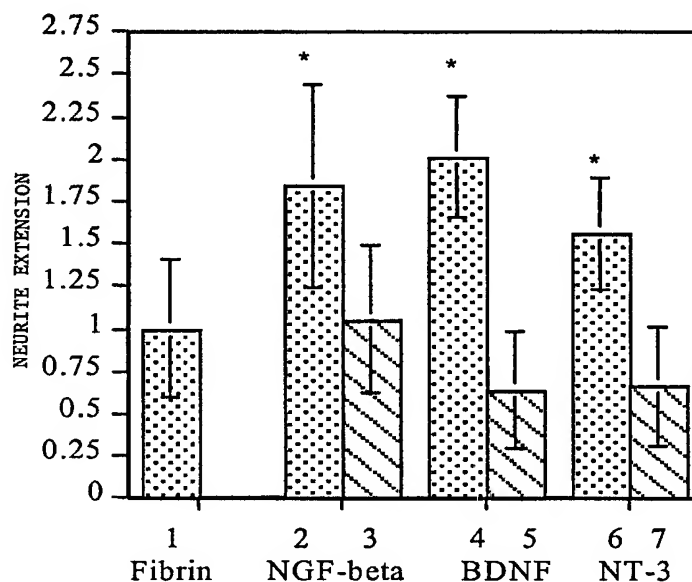
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A61K 47/48, 38/39, 38/18, 38/36, A61P 17/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/64481</b> <b>(43) International Publication Date:</b> 2 November 2000 (02.11.00)
<b>(21) International Application Number:</b> PCT/IB99/00800 <b>(22) International Filing Date:</b> 22 April 1999 (22.04.99) <b>(71) Applicant:</b> ETH-ZURICH AND UNIVERSITY OF ZURICH [CH/CH]; Moussonstrasse 18, CH-8044 Zurich (CH). <b>(72) Inventors:</b> SAKIYAMA, Shelly, Elbert; Hochstrasse 9, CH-8044 Zurich (CH). HUBBELL, Jeffrey, A.; Unterdorfstrasse 22, CH-8126 Zurich (CH).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	

**(54) Title:** CONTROLLED RELEASE OF GROWTH FACTORS FROM HEPARIN CONTAINING MATRICES**(57) Abstract**

Matrices that support cell adhesion and growth are disclosed that deliver low heparin-binding affinity growth factor protein peptides in a controlled manner. These matrices comprise covalently or non-covalently bound heparin or heparin-like polymers, which serve to sequester the low heparin-binding affinity growth factor protein peptides within the matrix. The controlled release of some low heparin-binding affinity growth factor or peptides thereof occurs by degradation of some matrix component or dissociation of the low heparin-binding affinity growth factor protein peptides from the bound heparin. This differs from many controlled delivery devices in that release is not controlled solely by diffusion, and the rate of release may therefore be regulated by altering the rate of degradation of the matrix component or the amount of heparin bound within the matrix.

The controlled release of such low heparin-binding affinity growth factor proteins such as NGF $\beta$ , NT-3 and BDNF, is demonstrated. The invention also identifies basic domains that can be utilized to identify other low heparin-binding affinity growth factor protein peptides useful in delivery as part of the matrices described herein.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## CONTROLLED RELEASE OF GROWTH FACTORS FROM HEPARIN CONTAINING MATRICES

**FIELD OF THE INVENTION**

The present invention relates to the field of three-dimensional matrices that  
5 contain pharmacologically active molecules, particularly growth factors. The  
invention also relates to the use of growth factors or proteins in a matrix designed to  
promote cell and tissue growth. The invention further relates to the use of growth  
factors with low heparin-binding affinity. In addition, the invention relates to the  
field of articles of manufacture useful as implantable devices and wound dressings as  
10 the matrix of the invention is designed to be used in conjunction with such devices to  
provide protracted and controlled release of growth factor, thus promoting wound  
healing in the patient.

**BACKGROUND**

Many growth factors are thought of as "heparin-binding" growth factors.  
15 Families with one or more members that bind heparin include fibroblast growth  
factors and bone morphogenetic proteins (BMPs) (1, 2). Additional growth factors  
that bind heparin include transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), interleukin-8,  
neurotrophin-6, vascular endothelial cell growth factor, heparin-binding epidermal  
growth factor, hepatocyte growth factor, connective tissue growth factor, midkine,  
20 and heparin-binding growth associate molecule (3-11). These factors have shown the  
potential to enhance healing in many different types of tissue including vasculature,  
skin, nerve, and liver.

Controlled delivery devices based on heparin-affinity of these growth factors  
have been designed previously (12-14). These drug delivery devices have previously

been used to deliver "heparin-binding" growth factors. Such "heparin-binding" growth factors are typically considered to be those which bind to heparin with a relatively high affinity, often characterized by elution from heparin-affinity columns at NaCl concentrations well above physiological levels (>140 mM). In such delivery systems, the heparin-binding affinity of the growth factor is usually used to sequester the growth factors to immobilized heparin of some form. For example, Edelman et al. have used heparin-conjugated Sepharose beads to bind basic fibroblast growth factor (bFGF) and then encapsulated the beads with alginate (12, 19). These beads serve as reservoirs that release bFGF slowly based on the binding and dissociation constants of bFGF and heparin.

The delivery of "non-heparin-binding growth factors" has previously required release methods for delivery typically based on diffusion-controlled release of the factors from porous materials (15-18). There remains a need in the medical arts for a device that is capable of providing the release of low heparin-binding growth factors at a controlled and predictable rate in order to provide effective release of the factor over a clinically useful period during the wound healing process.

### **SUMMARY OF THE INVENTION**

In a general and overall sense, the present invention relates to the use of how non-heparin-binding growth factors in delivery techniques employing growth factors with heparin-affinity by utilizing low heparin affinity sequences present in many proteins. The particular growth factors employed as part of the invention have been found by the present inventors to possess a basic sequence at a site in the protein that is freely accessible in the proteins native conformation. This basic region may possess only relatively low heparin-affinity.

As used in the description of the present invention, "low-heparin-binding affinity" of a growth factor or peptide fragment thereof is defined as any protein, peptide, or derivative or combination thereof, that is capable of demonstrating the biological activity of a growth factor, and that has a relatively binding low affinity for binding heparin, and will elute from a heparin-affinity column at sub-physiological NaCl concentrations. Physiological levels of NaCl may be defined as about 140 mM NaCl. Herein the term "sub-physiological" levels of NaCl, therefore, may be further defined as from between about 25 mM to about 140 mM NaCl. Although low heparin-binding affinity growth factors elute from heparin-affinity columns at sub-physiological NaCl concentrations, their low affinity for heparin can still be used to sequester the protein or peptide to a matrix that contains heparin or a heparin-binding site.

By way of example, and in no way intending to be limited to any particular mechanism of action, the invention may be described as employing a matrix having growth factor proteins with a relatively high ratio of heparin-binding sites. A ratio of at least 1:1 heparin to growth factor must be used, but the greater the excess of heparin sites the slower the release. In this fashion, primarily "non-heparin-binding" growth factors or peptide fragments thereof with relatively low heparin-binding affinity can be bound to a heparin-decorated matrix. These matrices can then serve as reservoirs containing the growth factor or factors to be delivered. The dissociation kinetics of low affinity heparin-binding proteins are relatively fast, but the high number of binding sites allows rebinding of the growth factor before it can diffuse out of the matrix. Release can occur by diffusion of the growth factor out of the matrix prior to rebinding, or it can occur if the growth factor encounters a cell surface

receptor before rebinding to a heparin site. In this fashion, release of the growth factor or bioactive fragment thereof can be sustained, and continue to foster improved healing.

In a general and overall sense, the present invention describes in at least one aspect a specially designed matrix that provides for the release of growth factors or bioactive fragments thereof. The growth factor is defined as having low binding affinity for heparin. The matrix, more particularly, may be defined as comprising a substrate capable of providing attachment of heparin, a heparin-like polysaccharide, or a heparin-like polymer, and a growth factor or peptide fragment thereof having a basic domain that binds heparin with low affinity.

The characteristic of the growth factor or peptide fragment thereof as binding to heparin with low affinity may be further described as a peptide/protein that will elute from a heparin affinity column at an NaCl concentration of about 25 mM to about 140 mM.

The "low heparin-binding affinity" growth factor or peptide fragment thereof may be further defined as comprising a length of about 8 to 30 amino acid residues. This sequence of amino acid residues, in some embodiments, may be defined as comprising at least 2 basic amino acid residues, a ratio of basic to acidic amino acid residues of at least 2, and a ratio of hydrophobic amino acid residues to basic amino acid residue of at least 0.67. The growth factor or fragment thereof elutes from a heparin affinity column at less than 140 mM or at about 25 to about 140 mM NaCl.

For purposes of this application, basic amino acids may be defined as K (lysine) or R (arginine). The acidic amino acid residues may be further defined as D (aspartic acid) or E (glutamic acid). The hydrophobic amino acid residues may be

defined as A (alanine), V (valine), F (phenylalanine), P (proline), M (methionine), I (isoleucine), or L (leucine). For purposes of this application, C (cysteine) that are involved in a disulfide bridge are also considered hydrophobic.

By way of example, the low heparin-binding affinity growth factor or a peptide fragment thereof as defined in the invention comprises neurturin, persephin, IGF-1A, IGF-1 $\beta$ , EGF, NGF $\beta$ , NT-3, BDNF, NT-4, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 4, or a peptide fragment of any one of these. Other growth factors may be found which contain similar basic domains that are not enumerated here. The matrix itself may also comprise any of a variety of materials, such as fibrin, collagen, hyaluronic acid, or a synthetic polymer hydrogel. By way of example, the synthetic polymer hydrogel may be a poly (ethylene glycol) hydrogel or a derivative thereof. Other synthetic polymer hydrogels may be used apart from those enumerated here.

The peptides of the invention that bind heparin with high affinity have a characteristic amino acid domain that will not elute from a heparin-affinity column at less than 140 mM NaCl. While many potential peptides exist, the inventors have identified several peptide sequences in particular. These are exemplified in the amino acid sequences identified in SEQ. ID. NO.:1, SEQ ID. NO.: 2; SEQ ID. NO.: 3; SEQ ID. NO.: 4; and SEQ ID. NO.: 5. Many other peptides may be used apart from the specifically enumerated sequences here.

The heparin or heparin-like polysaccharides of the invention may be further characterized, at least in some embodiments, as having a molecular weight of at least 3,000 Daltons. It is contemplated that virtually any molecular weight heparin or heparin-like polysaccharide could be used in the practice of the invention of at least 3,000 Daltons, without any upper molecular weight limitation. For practical purposes

a molecular weight maximum of 10,000,000 may be considered. In particular applications, the heparin-like polysaccharide may be further defined as a polysaccharide having a molecular weight of at least 3,000 Daltons and having at least one negative charge per two saccharide rings and no more than one positive charge per ten saccharide rings.

Examples of heparin-like polysaccharide include dextran sulfate, chondroitin sulfate, heparin sulfate, fucan, alginate or derivatives thereof. The present inventors have found that particular preparations of the matrix that include a particular molar ratio of heparin to growth factor, such as a molar ratio of 1, may be employed in the practice of the invention. It has further been found that a matrix of the invention that includes a molar ratio of covalently-attached peptide having a binding domain that binds heparin with high affinity to heparin or a heparin-like polysaccharide of at least one is in some embodiments of the matrix a preferred ratio. These heparin and heparin-like polysaccharides may be either covalently attached to the substrate or immobilized via non-covalent interactions (i.e. electrostatically bound). Synthetic polymers may be designed that function in a heparin-like manner.

The substrate of the matrix as defined in the present invention may comprise fibrin, collagen, hyaluronic acid, a synthetic polymer gel, a mixture thereof, or any variety of synthetic derivatives thereof, that is capable of supporting the attachment of the types of peptides described there, and/or cells.

The matrix and various embodiments of the matrix described herein provide a multitude of advantages, particularly when employed at tissue sites where a wound healing response is desired. The growth factor or peptide fragment thereof provided in the matrix is released by the degradation of a component of the matrix, by the



disassociation of growth factor from the heparin or heparin-like polysaccharide, or by a combination of these mechanisms. In this manner, a more sustained and controlled, release of growth factor into the site where the matrix is implanted may be achieved. Methods for providing the controlled release of growth factor at a wound site in need thereof are provided by the present invention. The growth factor for such applications may include TGF- $\beta$ 3, which may be particularly useful in dermal healing. The invention provides various articles of manufacture, such as a vascular graft or shunt, or tissue replacement that includes the matrix defined in this disclosure. Such articles may in some embodiments be defined as implantable sterilized compositions. The heparin-binding peptide used in the present examples possesses high heparin affinity.

In addition to using protein matrices as the substrate of the delivery system, other types of matrices can be used. Synthetic polymer hydrogels, including hydrogels formed by photopolymerization or conjugate addition reactions, can be utilized as the substrate for the delivery device. This synthetic material may contain cell adhesion domains, substrates for enzymatic degradation or hydrolysis, heparin-binding domains, or covalently bound heparin. Through either the covalent or non-covalent attachment of heparin, such synthetic matrices can bind low heparin-binding affinity growth factor proteins and release them in a controlled manner. Release can occur by degradation of matrix components or dissociation of the low heparin-binding affinity growth factor proteins, just as in protein matrices.

The substrate for the delivery system can also include matrices of hyaluronic acid or hyaluronic acid derivatives. Such materials are commonly used and readily available. The addition of covalently or non-covalently bound heparin or heparin-like polysaccharides can be used to provide controlled delivery of low heparin-binding

affinity growth factor proteins. Release can occur by degradation of matrix components or dissociation of the low heparin-binding affinity growth factor proteins, just as in protein or synthetic polymer matrices.

In addition to heparin, other heparin-like polymers have similar binding  
5 affinity for heparin-binding proteins and peptides. Such heparin-like polymers include, for example, dextran sulfate, chondroitin sulfate, heparin sulfate, fucan and alginate (See Maaroufi, et al, 1997 (46) and Logeart, et al., (1997) (47). In addition, synthetic heparin-like polymers or polysaccharide derivatives also exist, which have similar binding affinity for heparin-binding proteins or peptides as heparin. Examples  
10 of heparin-like polysaccharide derivatives include dextran derivatives such as those made by de Raucourt, et al., (1998) (48) and Bagheri-Yamand, et al., (1998) (49). Examples of heparin-like synthetic polymers include those by Silver, et al., (1992) (50). For the purposes of this invention, usage of the term "heparin" is considered to include all heparin-like polymers and polysaccharides including those described  
15 above.

(1) Amino acid three-letter and one-letter abbreviations:

<u>Abbreviation</u> <u>3 letter</u>	<u>Abbreviation</u> <u>1 letter</u>	<u>Amino Acid Name</u>
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Cys	C	Cysteine
Glu	E	Glutamic Acid
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

The following sequences are referenced in the description of the present invention.

SEQ ID NO: 1	ATIII domain	K(βA)FAKLAARLYRKA
SEQ ID NO: 2	PF4 domain	YKKIIKKL
SEQ ID NO: 3	NCAM domain	KHKGRDVILKKDVR
SEQ ID NO: 4	ATIII domain modified	R(βA)FARLAARLYRRA
SEQ ID NO: 5	bFGF domain	KDPKRLYRSRKY
SEQ ID NO: 6	NGF basic domain	CVLSRKAVRRA
SEQ ID NO: 7	NT-3 basic domain	CALSRKIGRT
SEQ ID NO: 8	BDNF basic domain	CTLTIKRGR

### **BRIEF DESCRIPTION OF THE DRAWINGS**

#### **Fig. 1**

Effect of matrix bound basic fibroblast growth factor on neurite extension

5 from dorsal root ganglia in three-dimensional fibrin gels. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. \* denotes  $p < 0.05$  versus unmodified fibrin. The soluble treatments contain bFGF, but not heparin or heparin-binding peptide. These treatments show no difference from unmodified fibrin suggesting that the washing protocol used was sufficient to remove unbound growth  
10 factor. The results also suggest that peptide, heparin, and growth factor is all necessary components of the controlled release system.

Bar 1, fibrin; Bar 2, with Peptide + heparin; Bar 3, 0.1,  $\mu\text{g/ml}$  bound; Bar 4, 1.0  $\mu\text{g/ml}$  bound; Bar 5, 1.0  $\mu\text{g/ml}$  soluble; Bar 6, 5.0  $\mu\text{g/ml}$  bound; Bar 7, 5.0  $\mu\text{g/ml}$  bound; Bar 8, 1.0  $\mu\text{g/ml}$  culture; Bar 9, VEGF 1.0  $\mu\text{g/ml}$ ; Bar 10, 1.0  $\mu\text{g/ml}$  bound to  
15 50% peptide; Bar 11, 1  $\mu\text{g/ml}$  + soluble ATIII; Bar 12, 1  $\mu\text{g/ml}$  + heparin (no peptide). "Bound" refers to fibrin, with Peptide and heparin. "Soluble" refers to fibrin.

#### **Fig. 2**

Effect of matrix bound NGF- $\beta$ , NT-3 and BDNF on neurite extension from

20 dorsal root ganglia in three-dimensional fibrin gels. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. \* denotes  $p < 0.05$  versus unmodified fibrin. The soluble treatments contain growth factor, but not heparin or heparin-binding peptide. These treatments show no difference from unmodified fibrin

suggesting that the washing protocol used was sufficient to remove unbound growth factor.

Bar 1, fibrin; Bar 2, NGF bound at 0.1 µg/ml; Bar 3, NGF soluble at 0.1 µg/ml; Bar 4 BDNF bound at 0.1 µg/ml; Bar 5, BDNF soluble at 0.1, µg/ml bound; Bar 6, NT-3 bound at 1.0 µg/ml; Bar 7, NT-3 soluble at 0.1 µg/ml bound. "Bound" refers to fibrin, with Peptide and heparin. "Soluble" refers to fibrin.

### Fig. 3

Ability of matrix bound NGF-β to promote neurite extension from dorsal root ganglia in three-dimensional fibrin gels as a function of wash time prior to cell seeding. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. \* denotes p<0.05 versus unmodified fibrin. The soluble treatments contain growth factor, but not heparin or heparin-binding peptide. This assay demonstrates that the matrix bound NGF-β maintains its activity for 4 days while the unbound NGF-β is not active after 1 day of washing.

--■-- fibrin; --◆-- ngf bound (100 ng/ml); --●-- ngf soluble (100 ng/ml)

### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions useful in promoting the controlled release of low affinity-heparin-binding growth factors having a low heparin-binding domain of about 8 to about 30 basic amino acids in length.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be

considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

**EXAMPLE 1 -HEPARIN-AFFINITY CHROMATOGRAPHY OF  
HEPARIN-BINDING AND LOW AFFINITY HEPARIN-BINDING  
GROWTH FACTORS**

10

Heparin-affinity chromatography is a method commonly used to determine the relative affinity of heparin-binding proteins. If a protein elutes at NaCl concentrations near or below physiological level (approximately 140 mM) it is not to be considered "heparin-binding" for purposes of the description of the present invention. This is because the growth factors would dissociate rapidly from heparin *in vivo*.

15

The relative affinity for heparin of proteins was determined by heparin-affinity chromatography, using a TSK-GEL Heparin-5PW (7.5 cm x 7.5 mm ID) column (TosoHass, Stuttgart, Germany). Samples of the protein were injected in 20 mM Tris, pH 7.4, 0.05 M NaCl. Elution was accomplished by running a gradient of NaCl from 0.05 M to 2.0 M over 30 min, and the NaCl concentration at which elution was observed was taken as a measure of the heparin-binding affinity of the protein. The relative heparin-binding affinity of proteins not previously reported in the literature to be heparin-binding was determined and compared with proteins such as bFGF and antithrombin III, which are known to be strongly heparin-binding. The results are shown in Table 1.

20

25

The two "non-heparin-binding" growth factors, TGF- $\beta$ 2 and NGF $\beta$  both eluted at sub-physiological NaCl concentrations, suggesting that they have relatively

low heparin-binding affinity, and will rapidly dissociate from heparin under physiological conditions. The two heparin-binding proteins, antithrombin III and bFGF, elute at NaCl concentrations that are much greater than physiological levels.

Others have also used heparin to increase the activity of and prevent the degradation of growth factors. Schroeder et al. (20) used heparin to increase the stability of TGF- $\beta$ 1 and prevent loss of activity. They have also attached heparin to collagen and employed the heparin-TGF- $\beta$ 1 complex in collagen gels to show that such heparin-based systems can improve growth factor activity in vivo by controlled release (21). TGF- $\beta$ 1 is known to possess strong heparin binding affinity. However, such heparin-based release systems have not been tested previously with growth factors, which are considered to have low heparin-binding affinity (those which are characterized by elution from heparin-affinity columns at sub-physiological NaCl concentrations).

Analysis of the primary sequence of proteins by the present inventors, including growth factors, has revealed that the primary sequence may contain regions that are basic in nature and as that the basic residues are commonly flanked by hydrophobic residues. These sequences are generally of a similar nature to the XBBXB heparin-binding consensus (where X is a hydrophobic residue and B is a basic residue) the heparin-binding consensus was described by Cardin and Weintraub (22). However, the exact sequence of the basic regions vary from protein to protein. The three-dimensional structure for many proteins is also available, which allows the location of basic regions to be determined. In order for basic regions to be useful for sequestration of a protein, they must be located on the surface of the protein.

Frequently, such regions are observed to occur in the amino or carboxy terminus of the protein.

- 5 Table 1: NaCl concentration required to elute proteins from a heparin-affinity column.

Protein	[NaCl] require to elute peptide
bFGF	2.0 M
antithrombin III	1.58 M
TGF- $\beta$ 2	0.05 M
NGF $\beta$	0.05 M

#### EXAMPLE 2 - *IN VITRO* MODEL FOR BIOACTIVITY - NEURITE EXTENSION

10

The present example is provided to describe the model for assaying *in vitro* bioactivity of materials described herein.

A three-dimensional fibrin gel *in vitro* model for assay neurite extension. Fibrinogen was dissolved in water and dialyzed into Tris-buffered saline (TBS) at pH  
15 7.4 for 24 hr. The concentration of fibrinogen was determined by measuring the absorbance at 280 nm. Fibrin gels were made containing a final concentration of 3.5 mg/ml fibrinogen, 2.5 mM Ca<sup>++</sup>, and 2 NIH units/ml thrombin in TBS. The polymerization mixture was incubated for 60 min at 37 °C, 95 % relative humidity, and 5% CO<sub>2</sub>.

20 After polymerization, 1 ml TBS was added to each well. The gels were washed 5 times over 24 hr, with the first 4 wash solutions consisting of TBS and the



last solution consisting of modified neural basal medium. Dorsal root ganglia (DRGs) were dissected from day 8 chick embryos and placed in Hanks-buffered salt solution. The ganglia were placed inside the fibrin gels (one per well) and the gels were incubated for 60 min at 37 °C, 95 % relative humidity, and 5% CO<sub>2</sub>. After 60 min, 1 ml of modified neural basal medium was added to each well. The media was changed at 24 hr. Bright field images of the ganglia were taken at 24 and 48 hr. The images were analyzed to determine the average length of neurite extension, which was calculated to be the area of an annulus between the DRG body and the outer halo of extending neurites, as shown by Herbert et al. (1996) (23). Neurite extension for each experiment was normalized by the average neurite extension through unmodified fibrin gels from the same experiment and time point. Results are shown in Figure 1, Bar 1. This study demonstrates the utility of the invention as a cell support and growth material that will enhance neurite extension in three-dimensions. This is also demonstrated of the utility the invention would have for promoting cell growth and neurite extension under conditions found *in vivo*.

### **EXAMPLE 3 -RELEASE OF BIOACTIVE NEUROTROPHINS FROM HEPARIN-BASED DELIVERY SYSTEM**

An Example of a growth factor that is not considered to be heparin-binding growth factors, but that contains a basic sequence is nerve growth factor  $\beta$  (NGF $\beta$ ) growth factor. This growth factor is considered to be non-heparin-binding and has even been used as a negative control for a heparin-binding protein in heparin-binding analysis (See Lee and Lander (1991) (24). However, NGF contains a basic domain at its C-terminus amino acid position (230-241 of human NGF beta) consisting of the following residues: CVLSRKAVRRA (SEQ ID NO:6). Similar basic domains can be found in the carboxy termini of neurotrophin-3 (NT-3, CALSRKIGRT, 248-257 of

human NT-3, SEQ ID NO: 7) and brain-derived neurotrophic factor (CTLTIKRGR, 238-247 of human BDNF, SEQ ID NO: 8).

The present example demonstrates that NGF $\beta$ , NT-3 and BDNF can be delivered in a sustained manner from heparin-containing fibrin matrices that contain non-covalently immobilized heparin. This system consists of covalently immobilized heparin-binding peptides cross-linked to the fibrin matrix by factor XIIIa (See Schense and Hubbell (1999) (25) and heparin, which is non-covalently attached to these heparin-binding peptides. These materials have been shown by the present inventor to effectively deliver heparin-binding growth factors, such as basic fibroblast growth factor. (U.S. Serial No. 09/141770, specifically incorporated herein by reference). With this system, a bi-domain peptide is synthesized to comprise a heparin-binding sequence in one domain and a substrate for the coagulation enzyme factor XIIIa in the other domain (Schense and Hubbell, (1999) (25)). This enzyme covalently attaches the substrate domain of the bi-domain peptide to the fibrin network during coagulation, thereby also immobilizing the heparin-binding domain of the bi-domain peptide to the fibrin network. Heparin is also included into the coagulation mixture, and the heparin is thereby immobilized to the fibrin network by binding to the immobilized peptide.

The release system was first characterized with growth factors that demonstrate strong heparin binding affinity. The results of these studies are shown in Figure 1. Bar 1 shows the neurite extension through unmodified fibrin gels. Bar 2 shows that the release system, consisting of heparin and incorporated heparin-binding peptide, does not promote neurite extension without the addition of growth factor. Bars 3, 4 and 6 show the dose-response effect of matrix-bound bFGF, which enhances

neurite extension by up to 100% (bar 6). Bars 5 and 7 show that when bFGF is added during polymerization of the fibrin gel in the absence of the release system, it does not enhance neurite extension, presumably because it diffuses out of the fibrin too quickly. Bar 8 shows that the presence of bFGF in the culture media promotes neurite extension similar to that of matrix bound bFGF at the same concentration. Bar 9 shows that VEGF does not enhance neurite extension, demonstrating that the growth factor bound must also show bioactivity in neural models to promote neurite extension. Bar 10 shows that when the amount of heparin-binding sites is decreased by 50%, the release of growth factor is not significantly affected. This deviation supports the contention that a high excess of growth factor binding sites is present. Bar 11 shows that when the peptide is cross-linked to the matrix, it will constitute a functional heparin-based delivery system. Bar 12 demonstrates that heparin and bFGF without the immobilized heparin-binding peptide do not constitute a functional delivery system and that heparin and immobilized peptide will enhance and sustain the sustained release of growth factor. These results demonstrate that the delivery system is capable of sustained release of a heparin binding growth factor in an active form.

The ability of heparin-containing fibrin matrices to deliver low-heparin-binding growth factors has been tested using NGF- $\beta$ , NT-3 and BDNF, all members of the neurotrophin family. Fibrin gels were made as described in Example 2, except for the addition of peptide, heparin and neurotrophin described below. Fibrin gels were made containing a final concentration of 3.5 mg/ml fibrinogen, 2.5 mM  $\text{Ca}^{++}$ , 2 NIH units/ml thrombin, 0.25 mM peptide, 0.125 mM heparin, and 0.1  $\mu\text{g/ml}$  of the neurotrophin to be tested. Otherwise, the assay was performed as described in

Example 2. The results are shown in Figure 2. The fibrin group represents the behavior of normal fibrin and was the control data set to which neurite extension was normalized. These ganglia were cultured in the presence of 20 ng/ml NGF. All other treatments contained no growth factors in the media. For each of the three neurotrophins tested, neurite extension was enhanced only when the heparin-based delivery system of peptide and heparin was present and bound to the matrix ("bound" bars). The addition of neurotrophin alone ("soluble" bars) without peptide or heparin did not enhance neurite extension presumably because the factor diffused out of the fibrin too quickly. The materials shown in Figure 2 differ from those in Figure 1 in that "low-heparin-binding affinity" growth factors were released in Figure 2, while the factors released in Figure 1 are considered strongly heparin-binding. This distinction is clearly demonstrated in Example 1, in which the heparin-binding growth factor (bFGF) eluted from heparin above physiological NaCl concentrations, whereas the low and non-heparin-binding growth factors (NGF- $\beta$ , and TGF- $\beta$ 2) eluted at sub-physiological NaCl concentrations. In both cases, the heparin-based delivery systems were able to deliver bioactive growth factors in a controlled manner. This demonstrates, surprisingly, that non-heparin-binding growth factors can be released in a sustained manner from heparin-containing matrices.

To determine how long the release of low-affinity growth factors could be sustained, NGF- $\beta$ -containing gels were washed for extended time periods prior to cell seeding. In each case, the gels were washed thoroughly with TBS. The results are shown in Figure 3. The bioactivity of the growth factor present after 4 days is the same as after 1 day of washing. After 1 week of washing, the bioactivity of the growth factor released is decreased.

These results demonstrate that NGF- $\beta$ , NT-3 and BDNF are being sequestered by heparin and that the increase in outgrowth versus unmodified fibrin is due to matrix bound growth factor, rather than free growth factor. This demonstrates that such heparin-based materials can be used to sequester proteins with low heparin-binding affinity, which contain exposed basic regions. In this three-dimensional fibrin matrix, heparin was immobilized at approximately 380  $\mu\text{g/ml}$ . This material demonstrated NGF release over approximately one week. There are situations when release for as little as a day would be therapeutically useful. Thus a useful amount of immobilized heparin would be at least 95  $\mu\text{g/ml}$ , with higher amounts leading to release sustained over longer periods.

The results shown here demonstrate that "non-heparin-binding growth factors", such as NGF $\beta$ , BDNF and NT-3, can be released in a controlled manner from heparin-based drug delivery systems based on their low affinity for heparin. These proteins can be sequestered within three-dimensional materials containing immobilized heparin based on basic domains found in the proteins. Furthermore, the growth factors released from these systems retain bioactivity in *in vitro* models as shown above. This demonstrates other low heparin-binding affinity growth factor proteins containing similar types of basic domains could be released from heparin-based delivery systems in a similar manner.

#### 20                    **EXAMPLE 4 - MEMBERS OF THE TGF- $\beta$ FAMILY**

The approach to sequence analysis described above can be applied to growth factors from other families as well. A list of growth factors and their sequences is shown in Table 2, which shows domains that may have low heparin-binding affinity and could be released from heparin-based delivery systems such as those described

above. These factors include some members of the TGF- $\beta$  family, namely TGF- $\beta$ 2, TGF- $\beta$ 3, and TGF- $\beta$ 4. TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 4 are members of a family, which contains one strongly heparin-binding growth factor, TGF- $\beta$ 1. However, other members of the TGF- $\beta$  family have been reported in the literature to have lower heparin-binding affinity, such as TGF- $\beta$ 3 (See Lyon et al (1997) (26). TGF- $\beta$ 1 has been shown to be heparin-binding at physiological ionic strength, i.e. at 140 mM NaCl. TGF- $\beta$ 3 lacks a key charge and has been demonstrated to be "non-heparin-binding" at physiological conditions. Heparin-affinity chromatography on TGF- $\beta$ 2 by the present inventors demonstrates that it also possesses low-heparin-binding affinity under physiological conditions. The basic domain in each of these growth factors, which could potentially interact with heparin, is underlined in the list of sequences given in Table 2.

Some members of a growth factor family may be heparin-binding, while others are not, as is demonstrated by the TGF- $\beta$  family. Despite the low-heparin-binding affinity of such "non-heparin-binding" growth factors, they may still be released in a controlled manner from heparin-based delivery systems if they contain a basic domain and the number of heparin-binding sites present in the system is in relatively greater excess to the amount of growth factor to be bound.

#### **EXAMPLE 5 - OTHER "NON-HEPARIN-BINDING" GROWTH FACTORS WHICH CONTAIN BASIC DOMAINS**

Other families of growth factors also contain growth factors which are not reported in the literature to be heparin-binding, but which contain basic domains(Shown in Table 2.

Table 2: GROWTH FACTOR SEQUENCES

TGF-beta1	ALDTNYCFSS TEKNCCVRQL YIDFRKKDLGW KWIHEPKGYH ANFCLGPCPY IWSLDTQYSK VLALYNQHNP
TGF-beta2	ALDAAYCFRN QQDNCCLRPL YIDFKRRDLGW KWIHEPKGYN ANFCAGACPY LWSSTQHSR VLSLYNTINP
TGF-beta3	ALDTNYCFRN LEENCCVRPL YIDFRQDLGW KWIHEPKGYG ANFCSGPCPY LRSADTTHTST VLGLYNTLNP
TGF-beta1	GASAAPCCVP QALEPLIVY YVGRKPKVEQ LSNMIVRSCK CS
TGF-beta2	EASAPCCVS QDLEPLTILY YIGKTPKIEQ LSNMIVKSCK CS
TGF-beta3	EASAPCCVP QDLEPLTILY YVGRTPKVEQ LSNMIVKSCK CS
TGF-beta4	FSQS FREVAGRFLA SEASTHLLVF GMEQRLPPNS ELVQAVLRLF QEPVPQALH RHGRLLSPAAP KARVTVEVLV RDDGSRNTSL IDSRLSVHE SGWKAQDVTE AVNFWQQLSR PPEPLLVQVS VQREHLGPLA SGAHKLVRF SQGAPAGLGE PQLELHTLDL RDYGAQGD CD PEAPMTEGTR CCRQEMYIDL QGMKWAKNWW LEPPGFLAYE CVGTCQQPPE ALAFNWPFLG PRQCIASETA SLPMTVISKE GGRTRPQVVS LPNMRVQKCS CASDGAALVPR <u>RLQHRPWCIH</u>
GDNF Neurturin	SPD KQMAVLP <b>RRE</b> RNRQAAAAANP ENSRGKRRG QRGKNRGCVL TAIHLNVTDL GLGYETKEEL IFRYCSGSCD LGA <b>RPCGLRELEV</b> RVSELGLGYA SDETVLFRYC AGACEAAARV YDLGLRRLRQ <b>RRRLRRERVR</b> AQPCCRPATAY
GDNF Neurturin	AAETTYDKIL KNL <b>SRNRRLV</b> SDKVGQACCR PIAFDDDLDF LDDNLVYHIL <b>RKHSARK</b> CGC I EDEVSFLDAH <b>SR</b> YHTVHEL SARECAY
NT-4	GVSETAPASR <b>RG</b> ELAVCDVSG
NGF-beta BDNF	SSSHPIFHRG EFSVCDSSV WVGDKTTATD IKGKEVMVLG EVNINNSVK QYFETKCRDP NPVDSGCRGID HSDPARRGEL SVCDISEWV TAADKKTAVD MSGGTVTVLE KVPVSKGQLK QYFETKCNP MGYTKEGCRGID YAEHKSHRGEY SVCDSESLW TDKSSAIDIR GHQVTVLGE IKTGNSPVK QYFETRCKE ARPVKNGCRGID
NT-3	WVTD <b>RRR</b> TAVD LRGREVEVLG EVPAAGGSP L RQYFETRCK ADNAEEGGPG AGGGGCRGVD <b>RR</b> HHWVSECVD
NT-4	SKHWNSYCTT THTFV <b>KALTM</b> DGKQAAWRF IRIDTACVCL <b>LSRKAVRRA</b> <b>KR</b> HWNSQCRT TQSYVRALTM DSKKRIGWRF IRIDTSCVCT <b>LTIKRGR</b> DKHWNSQCKT SQTYYRALTS ENNKLVGWRW IRIDTSCVCA <b>LSRKIGRI</b> <b>RR</b> HWVSECKA KQSYVRALTA DAQGRVGRW IRIDTACVCT <b>LSRTGRA</b>
IGF-1A	GP ETLCGAELVD ALQFVCGDRG FYFNKPTGYG SSSRRRAPQTG INDECCFRSC <b>DLRR</b> LEMYCA PL <b>KPAKSA</b>
IGF-1B	GP ETLCGAELVD ALQFVCGDRG FYFNKPTGYG SSSRRRAPQTG INDECCFRSC <b>DLRR</b> LEMYCA PL <b>KPAKSA</b>
EGF	NSDSECLSH DGYCLHDGVC MYTEALDKYA CNVVGTYGE <b>RCOYRDLKWW</b> ELR

Table 2: Sequences of Low and High heparin-Binding Affinity Growth Factor Proteins.

Basic domains of low heparin-binding affinity growth factor proteins are underlined and basic amino acid residues (K or R) are shown in bold. Analysis of the primary protein of growth factors, such as those shown in Table 2 can be used to identify basic domains.

By analyzing the sequences of the TGF $\beta$  family and the neurotrophin family, one skilled in the art can observe a pattern in the basic domains underlined in Table 2. From this pattern observed in the basic domains of low heparin-binding affinity proteins, an approximate formula was developed to identify similar basic domains in other proteins. The formula defines a basic domain to be of length about 8 to 30 amino acid residues, comprising at least 2 basic amino acid residues, with a ratio of basic to acidic amino acid residues to at least 2, and a ratio of hydrophobic amino acid residues of at least 0.67. Secondary and tertiary protein structure also influences the heparin-binding affinity of a basic domain within a protein or peptide, and for this reason the formula is approximate. Therefore, it is necessary to perform heparin-affinity chromatography or some other experimental technique to determine the relative heparin affinity of a protein or peptide. The term "low heparin-binding affinity protein" refers only to those proteins or peptides which elute from heparin-affinity chromatography at a NaCl concentration of less than about 140 mM. The formula for sequence analysis described above was applied to the list of growth factors found in Table 2 and used to identify basic domains which could potentially bind to heparin or heparin-like polymers.

Low heparin-binding affinity protein should dissociate rapidly from heparin under physiological conditions. However, surprisingly the results shown in Fig. 3 demonstrate that such low heparin-binding affinity growth factor proteins can be released in a controlled manner from heparin-based delivery systems. This novel result suggests that other growth factors containing domains which meet the requirements of the formula described above may also be released in a controlled manner from heparin-based delivery systems. Examples of such growth factors are



shown in the sequence list in Table 2, and the basic domains of these growth factors are underlined.

Two members of the GDNF family, namely neurturin and persephin, both contain basic domains. GDNF is reported in the literature to be heparin-binding, but  
5 no reports have been made to date on the heparin-affinity of other members of the GDNF family (27). Neurturin and persephin, based on the analysis presented above, would appear to have sufficient heparin binding affinity to be releasable by the methods and materials of this invention.

IGF-1A and IGF-1B are members of the insulin-like growth factor  
10 family. Although there are extensive reports of insulin-like growth factor binding proteins binding to heparin, there is no documentation in the literature of IGF-1A or IGF-1B binding to heparin. Both of these proteins contain basic domains shown in Table 2. Based on the analysis presented above, would appear to have sufficient heparin binding affinity to be releasable by the methods and materials of this  
15 invention.

EGF is another growth factor, which contains a basic domain, shown in Table 2, but which is not reported in the literature to be heparin-binding. In fact, the existence of a related growth factor specifically referred to as heparin-binding EGF-like growth factor suggests that EGF does not possess high heparin-affinity. The  
20 literature suggests EGF is not heparin-binding based on sequence analysis (45). Based on the analysis presented above, it appears to the present inventors to have sufficient heparin binding affinity to be releasable by the methods and materials of this invention.

Despite the basic domains found in each of these proteins, their heparin-binding affinity is still weak and characterized by elution from heparin-affinity columns at sub-physiological NaCl concentrations (i.e. growth factors which elute between about 25 mM and 140 mM NaCl). For example, heparin-affinity chromatography was performed for NGF- $\beta$  and TGF- $\beta$ 2. In both cases the proteins were found to elute at 50 mM NaCl at pH 7.4 after several column volumes of buffer, which is well below the physiological NaCl concentration. However, *in vitro* studies demonstrate that heparin-based release systems can still be used to release NGF- $\beta$  in a controlled manner, in spite of its relatively low heparin-binding affinity.

All of the growth factors described in the example contain basic domains, but lack any literature reports of heparin-binding affinity. However, based on comparison of the sequences and the results demonstrated with other non-heparin-binding growth factors of the neurotrophin family, sustained release from heparin-based systems should be possible with virtually any growth factor having a basic domain having the characteristics of basic domains described above.

## BIBLIOGRAPHY

The following references are specifically incorporated herein by reference for the purposes indicated herein:

1. Presta, M., Satuto, M., Isacchi, A., Caccia, P., Pozzi, A., Gualandris, A., Rusnati, M., Bergonzoni, L., and Sarmientos, P. (1992) *Biochem Biophys Res Commun* 185: 1098-1107
2. Reddi, A. (1998) *Nature Biotechnology* 16: 247-252
3. McCaffrey, T., Falcone, D., and Du, B. (1992) *J Cell Physiol* 152: 430-440

4. Spillmann, D., Witt, D., and Lindahl, U. (1998) *J Biol Chem* 273: 15487-15493
5. Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Scharl, M., and Thoenen, H. (1994) *Nature* 372: 266-269
- 5 6. Tessler, S., Rockwell, P., Hicklin, D., Cohen, T., Levi, B., Witte, L., Lemischka, I., and Neufeld, G. (1994). *J Biol Chem* 269: 12456-12461
7. Kiguchi, K., Beltran, L., Rupp, T., and DiGiovanni, J. (1998) *Mol Carcinog* 22: 73-83
8. Kinosaki, M., Yamaguchi, K., Murakami, A., Ueda, M., Morinaga, T., and Higashio, K. (1998) *Biochim Biophys Acta* 1384: 93-102
- 10 9. Steffen, C., Ball-Mirth, D., Harding, P., Bhattacharyya, N., Pillai, S., and Brigstock, D. (1998) *Growth Factors* 15: 199-213
10. Kaneda, N., Talukder, A., Nishiyama, H., Koizumi, S., and Muramatsu, T. (1996) *J Biochem (Tokyo)* 119: 1150-1156
- 15 11. Nolo, R., Kaksonen, M., and Rauvala, H. (1996). *Eur J Neurosci* 8: 1658-1665
12. Edelman, E., Mathiowitz, E., Langer, R., and Klagsbrun, M. (1991) *Biomaterials* 12: 612-626
13. DeBlois, C., Cote, M.-F., and Doillon, C. (1994) *Biomaterials* 15: 665-672
- 20 14. Downs, E., Robertson, N., Riss, T., and Plunkett, M. (1992) *J Cell Physiol* 152: 422-429
15. Houle, J. and Johnson, J. (1989) *Neurosci Lett* 103: 17-23
16. Camarata, P., Suryanarayanan, R., Turner, D., Parker, R., and Ebner, T. (1992) *Neurosurgery* 30: 313-319

17. Powell, E., Sobarzo, M., and Saltzman, W. (1990) *Brain Res* 515: 309-311
18. Maysinger, D., Jalenjak, I., and Cuello, A. (1992) *Neurosci Lett* 140: 71-74
19. Edelman, E., Langer, R., Klagsburn, M., and Mathiowitz, E. (1992)  
*Controlled Release Systems Containing Heparin and Growth Factors*, MIT: USA
- 5 20. Schroeder, J., Bentz, H., and Estridge, T. (1997) *Affinity bound collagen  
matrices for the delivery of biologically active agents*, Collagen Corporation: USA.
21. Schroeder-Tefft, J., Bentz, H., and Estridge, T. (1997) *J Controlled Release*  
49: 291-298
22. Cardin, A. and Weintraub, H. (1989) *Atherosclerosis* 9: 21-32
- 10 23. Herbert, C., Bittner, G., and Hubbell, J. (1996) *J Comp Neurol* 365: 380-391
24. Lee, M. and Lander, A. (1991) *Proc Natl Acad Sci U S A* 88: 2768-2772
25. Schense, J. and Hubbell, J. (1999) *Bioconjug Chem* 10: 75-81
26. Lyon, M., Rushton, G., and Gallagher, J. (1997) *J Biol Chem* 272: 1800-  
18006
- 15 27. Lin, L.-F.H., Zhang, T., Collins, F., and Armes, L. (1994) *J Neurochem* 63:  
758-768
28. Besson, C, Saulnier J., Wallach, J. (1996) *Analytical Biochemistry* 237: 216-  
223
29. Coombs G., Bergstrom R., Madison, E., and Corey, D. (1998) *The Journal of*  
20 *Biological Chemistry* 237: 4323-4328
30. Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Shartl, M., and  
Thoenen, H. (1994) *Nature* 372: 266-269

31. Hata, A., Ridinger, D., Sutherland, S., Emi, M., Shuhua, Z., Myers, R., Ren, K., Cheng, T., Inoue, I., Wilson, D., Iverius, P., and Lalouel, J. (1993) *The Journal of Biological Chemistry* 268: 8847-8457
32. Haugen, P., McCarthy, J., Roche, K., Furcht, L., and Letourneau, P. (1992)  
5 *The Journal of Neuroscience* June 2034-2042
33. Kallapur, S. and Akeson, R. (1992) *Journal of Neuroscience Research* 33: 538-548
34. Kaneda, N., Talukder, A., Nishiyama, H., Koizumi, S., and Muramatsu, T. (1996) *J. Biochem* 119: 1150-1156
- 10 35. Kiguchi, K., Beltrán, L., Rupp, T., and DiGiovanni, J. (1998) *Molecular Carcinogenesis* 22: 73-83
36. Kinoshita, M., Yamaguchi, K., Murakami, A., Ueda, M., Morinaga, T., Higashio, K. (1998) *Biochimica et Biophysica Acta* 1384: 93-102
37. McCaffrey, T., Falcone, D., and Du, B. (1992) *Journal of Cellular Physiology*  
15 152: 430-440
38. Netzel-Arnett, S., Fields, G., Birkedal-Hansen, H., and Van Wart, H. (1991) *The Journal of Biological Chemistry* 266: 6747-6755
39. Nolo, R., Kaksonen, M. and Rauvala, H. (1996) *European Journal of Neuroscience* 8: 1658-1665
- 20 40. Smith, M., Shi, L. and Navre, M. (1995) *The Journal of Biological Chemistry* 270: 6440-6449
41. Spillmann, D., Witt, D., and Lindahl, U. (1998) *The Journal of Biological Chemistry* 273: 15487-15493

42. Steffen, C., Ball-Mirth, D., Harding, P., Bhattacharyya, N., Pillai, S., and Brigstock, D. (1998) *Growth Factors* 15: 199-213
43. Presta, et al. (1992) *Biochemical and Biophysical Research Communications*, 185: 1098-1107 (1992)
- 5 44. Zucker, M. and Katz, I., (1991) *Society for Experimental Biology and Medicine*: 693-702
45. Higashiyama S., Abraham J. A., Miller J., Fiddes, J. C., and Klagsbrun, M. (1991) *Science* 251 (4996): 936-9
46. Maaroufi, R. M., Jozefonwicz, M., Tapon-Brethaudiere J., Jozefonwicz, J.,  
10 Fischer, A. M., *Biomaterials* 1997, 18:359-366.
47. Logeart, D., Prrgent-Richard, S., Jozefonwicz, J., Letourneur, D., *Eur J Cell Biol* 1997, 74:376-384.
48. de Raucourt, E., Maurat, S., Chaubet, F., Maiga-Revel, O., Jozefonwicz, M., Fischer, A. M., *J. Biomed Mater Res* 1998, 41 :49-57
- 15 49. Bagheri-Yamand, R., Kourbali, Y., Mabilat, C., Morere, J. F., Martin, A., Lu, H., Soria, C., Jozefonwicz, J., Crepin, M., *Br. J. Cancer* 1998, 78:111-118.
50. Silver, J. H., Hart, A. P., Williams, E. C., Cooper S. L., Charef, S., Labarre, D., Jozefonwicz, M., *Biomaterials* 1992, 13:339-344.
51. U.S. Patent No. 5,830,700 Irani, Meher

What is claimed is:

1. A matrix comprising:
  - a substrate capable of providing attachment of a heparin-binding peptide;
  - 5 a peptide comprising a binding domain that binds heparin with high affinity;
  - heparin or a heparin-like polymer; and
  - a protein growth factor or peptide fragment thereof having a domain that binds heparin with low affinity.
2. The matrix of claim 1 wherein the growth factor or peptide fragment thereof  
10 that binds heparin with low affinity is further defined as eluting from a heparin-affinity column at a NaCl concentration of about 25 mM to about 140 mM.
3. The matrix of claim 1 wherein the domain of the growth factor or peptide fragment thereof is further defined as comprising a length of about 8 to 30 amino acid residues comprising at least 2 basic amino acid residues, a ratio of basic to acidic  
15 amino acid residues of at least 2, and a ratio of hydrophobic amino acid residues to basic amino acid residues of at least 0.67.
4. The matrix of claim 3 wherein the basic amino acid residue is K or R.
5. The matrix of claim 3 wherein the acidic amino acid residue is further defined as D or E.
- 20 6. The matrix of claim 3 wherein the hydrophobic amino acid residue is further defined as A, V, F, P, M, I, or L or C when C is involved in a disulfide bond.
7. The matrix of claim 1 wherein the growth factor or peptide fragment thereof is neurturin, persephin, IGF-1A, IGF-1 $\beta$ , EGF, NGF $\beta$ , NT-3, BDNF, NT-4, TGF- $\beta$ 2, TGF- $\beta$ 3, or TGF- $\beta$ 4.

8. The matrix of claim 7 wherein the growth factor or peptide fragment there is further defined as neurturin or a peptide fragment thereof.
9. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is further defined as persephin or a peptide fragment thereof.
- 5 10. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is further defined as IGF-1A or a peptide fragment thereof.
11. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is further defined as IGF-1 $\beta$  or a peptide fragment thereof.
12. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is  
10 further defined as EGF or a peptide fragment thereof.
13. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is further defined as NGF $\beta$  or a peptide fragment thereof.
14. The matrix of claim 1 wherein the growth factor or peptide fragment thereof is further defined as NT-3 or a peptide fragment thereof.
- 15 15. The matrix of claim 1 wherein the growth factor or peptide fragment thereof is further defined as BDNF or a peptide fragment thereof.
16. The matrix of claim 1 wherein the low heparin-binding affinity growth factor protein or peptide fragment thereof is further defined as NT-4 or a peptide fragment thereof.
- 20 17. The matrix of claim 1 wherein the low heparin-binding affinity growth factor protein or peptide fragment thereof is further defined as TGF- $\beta$ 2 or a peptide fragment thereof.



18. The matrix of claim 1 wherein the low heparin-binding affinity growth factor protein or peptide fragment thereof is further defined as TGF- $\beta$ 3 or a peptide fragment thereof.
19. The matrix of claim 1 wherein the low heparin-binding affinity growth factor  
5 protein or peptide fragment thereof is further defined as TGF- $\beta$ 4 or a peptide fragment thereof.
20. The matrix of claim 1 wherein the substrate comprises fibrin.
21. The matrix of claim 1 wherein the substrate comprises a synthetic polymer hydrogel.
- 10 22. The matrix of claim 1 wherein the peptide comprising a domain that binds heparin with high affinity is further defined as eluting from a heparin-affinity column at not less than 140 mM NaCl.
23. The matrix of claim 1 wherein the peptide comprising a domain that binds heparin with high affinity is further defined as comprising SEQ ID NO: 1, SEQ ID  
15 NO:2, SEQ ID NO:3, SEQ ID No:4, or SEQ ID NO:5.
24. The matrix of claim 1 wherein the heparin or heparin-like polymer has a molecular weight between about 3,000 and 10,000,000 Daltons.
25. The matrix of claim 1 wherein the heparin-like polymer is a polysaccharide having a molecular weight between about 3,000 and 10,000,000 Daltons, and having  
20 at least one negative charge per two saccharide rings and no more than one positive charge per ten saccharide rings.
26. The matrix of claim 1 wherein the heparin-like polymer is dextran sulfate, chondroitin sulfate, heparan sulfate, fucan, alginate, or a derivative thereof.

27. The matrix of claim 1 wherein the molar ratio of heparin or heparin-like polymer to growth factor is at least one.
28. The matrix of claim 1 wherein the molar ratio of covalently attached peptide having a binding domain that binds heparin with high affinity to heparin or a heparin-like polymer is at least one.
29. A matrix comprising:  
a substrate capable of providing attachment of heparin or a heparin-like polymer;  
heparin or a heparin-like polymer; and  
a growth factor or peptide fragment thereof having a domain that binds heparin with low affinity.
30. The matrix of claim 29 wherein the low heparin-binding affinity of the growth factor protein or peptide fragment thereof is further defined as eluting from a heparin-affinity column at a NaCl concentration of about 25 mM to about 140 mM.
31. The matrix of claim 29 wherein the growth factor protein or peptide fragment thereof comprises of a domain length of about 8 to 30 amino acid residues comprising at least 2 basic amino acid residues, a ratio of basic to acidic amino acid residues of at least 2, and a ratio of hydrophobic amino acid residues to basic amino acid residues of at least 0.67, which growth factor protein further elutes from a heparin-affinity column at a NaCl concentration less than about 140 mM.
32. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as neurturin, persephin, IGF-1A, IGF-1 $\beta$ , EGF, NGF $\beta$ , NT-3, BDNF, NT-4, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 4, or a peptide fragment thereof having a domain that binds heparin with low affinity.

33. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as neurturin or a peptide fragment thereof having a domain that binds heparin with low affinity.
34. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as persephin or a peptide fragment thereof having a domain that binds heparin with low affinity.
35. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as IGF-1A or a peptide fragment thereof having a domain that binds heparin with low affinity.
36. The matrix of claim 29 wherein the growth factor or peptide fragment thereof is further defined as IGF-1 $\beta$  or a peptide fragment thereof having a domain that binds heparin with low affinity.
37. The matrix of claim 29 wherein the growth factor or peptide fragment thereof is further defined as EGF or a peptide fragment thereof having a domain that binds heparin with low affinity.
38. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as NGF $\beta$  or a peptide fragment thereof having a domain that binds heparin with low affinity.
39. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as NT-3 or a peptide fragment thereof having a domain that binds heparin with low affinity.
40. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as BDNF or a peptide fragment thereof having a domain that binds heparin with low affinity.

41. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as NT-4 or a peptide fragment thereof having a domain that binds heparin with low affinity.
42. The matrix of claim 29 wherein the low heparin-binding affinity growth factor  
5 or a peptide fragment thereof is further defined as TGF- $\beta$ 2 or a peptide fragment thereof having a domain that binds heparin with low affinity.
43. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as TGF- $\beta$ 3 or a peptide fragment thereof having a domain that binds heparin with low affinity.
- 10 44. The matrix of claim 29 wherein the growth factor or a peptide fragment thereof is further defined as TGF- $\beta$ 4 or a peptide fragment thereof having a domain that binds heparin with low affinity.
45. The matrix of claim 29 wherein the substrate comprises fibrin.
46. The matrix of claim 29 where the substrate comprises collagen.
- 15 47. The matrix of claim 29 wherein the substrate comprises hyaluronic acid or a hyaluronic acid derivative.
48. The matrix of claim 29 wherein the substrate comprises a synthetic polymer hydrogel.
49. The matrix of claim 29 wherein the heparin or heparin-like polymer has a  
20 molecular weight between about 3,000 and 10,000,000 Daltons.
50. The matrix of claim 29 wherein the heparin-like polymer is a polysaccharide having a molecular weight between about 3000 and 10,000,000 Daltons and having at least one negative charge per 2 saccharide rings and no more than 1 positive charge per 10 saccharide rings.

51. The matrix of claim 29 wherein the heparin-like polymer is dextran sulfate, chondroitin sulfate, heparan sulfate, fucan, alginate, or a derivative thereof.

52. The matrix of claim 29 wherein a molar ratio of heparin or heparin-like polymer to growth factor or a peptide fragment thereof is included in the matrix of at least 1.

53. A matrix comprising:  
a substrate capable of supporting the attachment of a cell, wherein said substrate comprises heparin or a heparin-like polymer bound thereto; and  
growth factor or a peptide fragment thereof having a domain that binds heparin with low affinity,  
wherein under physiological conditions the low heparin-binding affinity growth factor is released by degradation of a component of the matrix or by dissociation of the growth factor from the heparin or heparin-like polymer.

54. The matrix of claim 53 wherein the substrate comprises fibrin.

55. The matrix of claim 53 wherein the heparin or heparin-like polymer is non-covalently attached to the substrate.

56. The matrix of claim 53 wherein the heparin or heparin-like polymers is covalently attached to the substrate.

57. A vascular graft comprising a matrix capable of supporting cell adhesion, said matrix comprising bound heparin or heparin-like polymer and a growth factor having low binding affinity for heparin.

58. An article for treatment of dermal wounds comprising a matrix capable of supporting cell adhesion, said matrix comprising bound heparin or heparin-like polymer and a growth factor having low binding affinity for heparin.

59. The article of claim 58, wherein the growth factor is TGF- $\beta$ 3.

60. An article of manufacture comprising a matrix capable of supporting cell adhesion, said matrix comprising bound heparin or heparin-like polymer and a growth factor or peptide fragment thereof having low binding affinity for heparin.

5 61. An implantable sterilized composition comprising a matrix capable of supporting cell adhesion, said matrix comprising bound heparin or a heparin-like polymer and a growth factor or peptide fragment thereof having low binding affinity for heparin.

62. A method for providing controlled release of growth factor comprising:

10 preparing a matrix comprising a growth factor having a domain with low affinity for binding heparin and bound heparin or heparin-like polymer; and  
placing the matrix on a wound in need thereof.

63. The method of claim 62, wherein the growth factor is released by degradation of a component of the matrix or by dissociation of the growth factor from the heparin  
15 or heparin-like polymer.

FIGURE 1

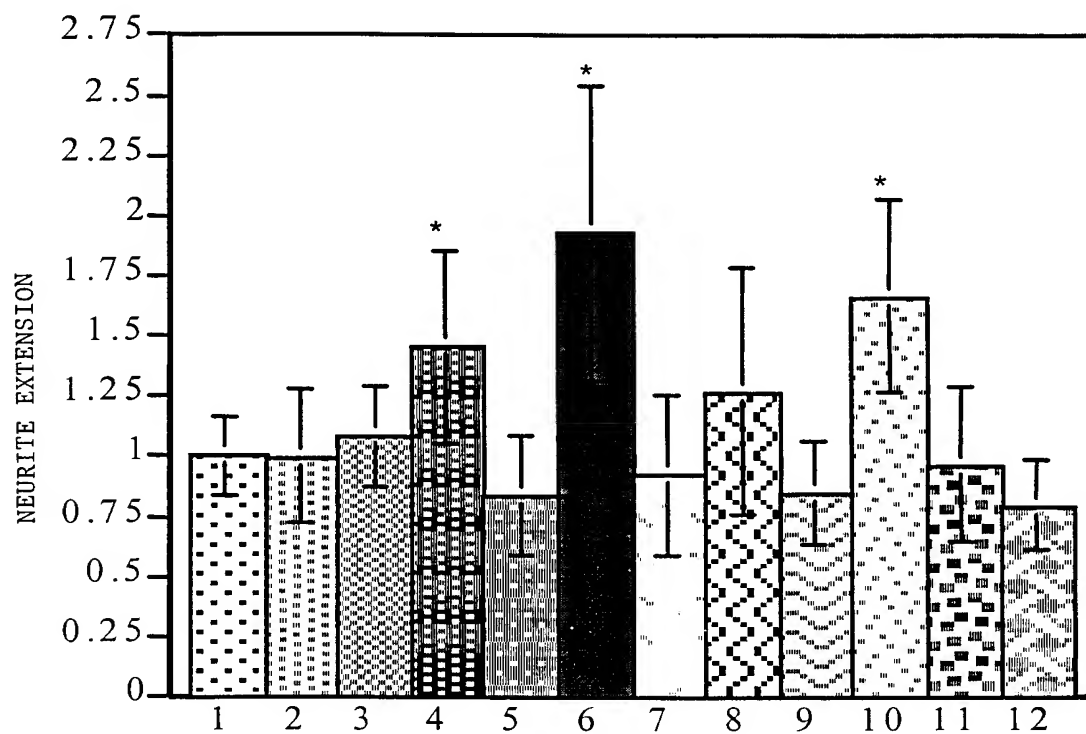


FIGURE 2

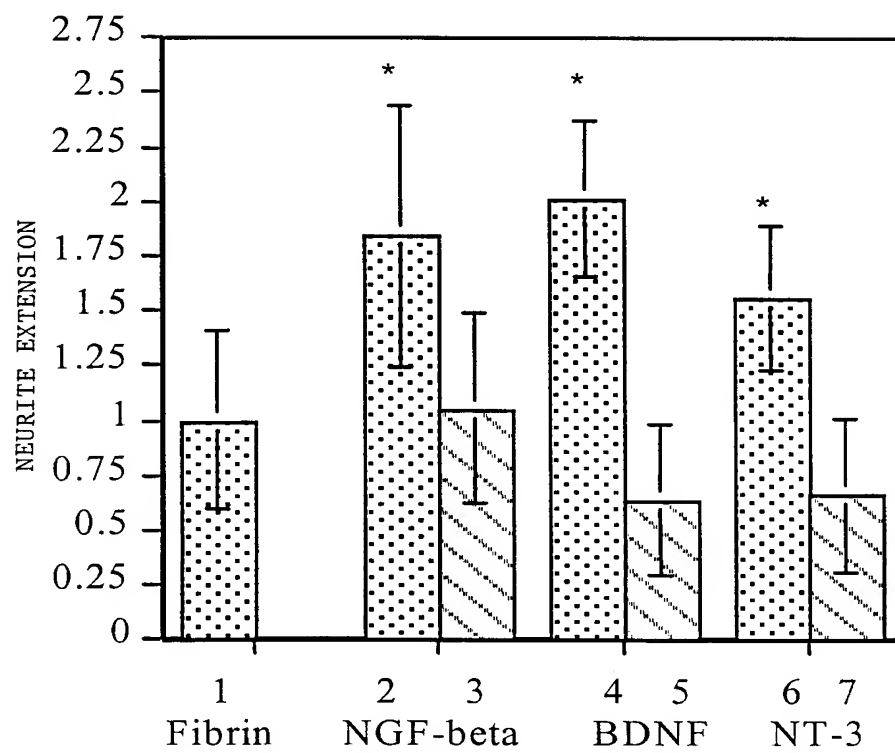
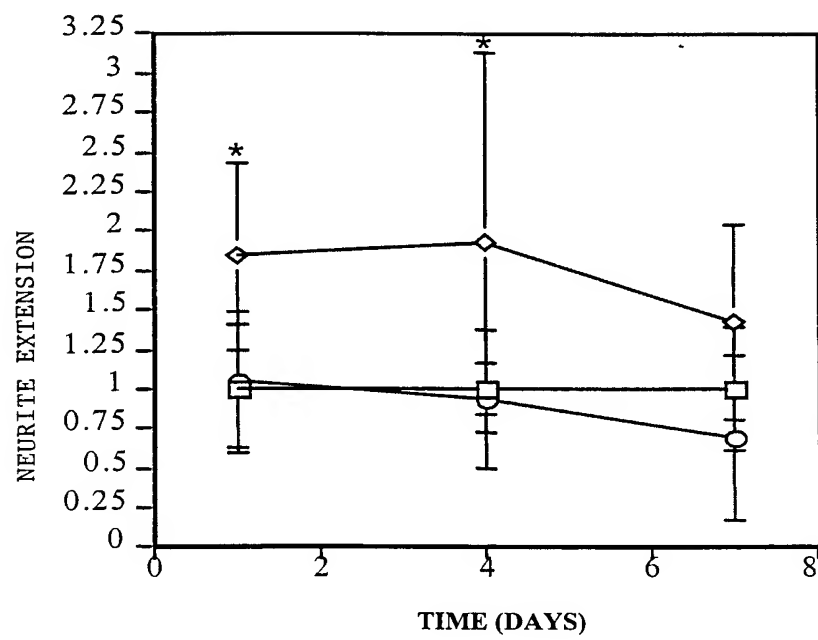




FIGURE 3



5

10

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/00800

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K47/48 A61K38/39 A61K38/18 A61K38/36 A61P17/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 16983 A (JOLLA CANCER RES FOUND) 6 June 1996 (1996-06-06) page 15; claims 4,6,8 ---	1-63
E	WO 99 54359 A (HUMPHRYS SIMON TROY ; GROPEP PTY LTD (AU); BALLARD FRANCIS JOHN (AU) 28 October 1999 (1999-10-28) abstract page 12, line 20-25; claims 3,4,31,60; examples 7,13 ---	1-63
E	WO 99 21588 A (GALLAGHER JOHN THOMAS ; PYE DAVID ALEXANDER (GB); CANCER RES CAMPAI) 6 May 1999 (1999-05-06) abstract; claims 1,8,37 --- -/--	1-63

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

2 June 2000

Date of mailing of the international search report

16.06.00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Gonzalez Ramon, N

## INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/IB 99/00800

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 09301 A (GREISLER HOWARD P ;AMERICAN NAT RED CROSS (US)) 11 June 1992 (1992-06-11) abstract; claims 3,4,7 ---	1-63
X	EP 0 838 219 A (SUMITOMO PHARMA) 29 April 1998 (1998-04-29) page 3 -page 4 page 5, line 25-33; claims 2-4 ---	1-63
X	EP 0 732 105 A (COLLAGEN CORP) 18 September 1996 (1996-09-18) abstract column 6, line 22-36; claims 2,3,6,9-11; examples 1,6 ---	1-63
E	DE 198 41 698 A (CURATIVE TECHNOLOGIES GMBH) 16 March 2000 (2000-03-16) abstract; claims 2,16,20,21; example 2 ---	1-63
E	WO 00 13710 A (SCIOS INC ;YANG BING (US); JENNINGS ROBERT N JR (US); PROTTER ANDR) 16 March 2000 (2000-03-16) abstract; claims 1,2,12,13 ---	1-63
T	SAKIYAMA S. E. ET AL: "Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering" FASEB, vol. 13, December 1999 (1999-12), pages 2214-2224, XP002128749 abstract; table 1 page 2223 ---	1-63
X	SCHROEDER-TEFFT J. A. ET AL: "Collagen and heparin matrices for growth factor delivery" JOURNAL OF CONTROLLED RELEASE, vol. 49, 1997, pages 291-298, XP000867263 abstract see conclusions ---	1-63
X	SCHENSE J. C. ET AL: "Cross linking exogenous bifunctional peptides into fibrin gels with factor XIIIa" BIOCONJUGATE CHEMISTRY, vol. 10, no. 1, 12 August 1998 (1998-08-12), pages 75-81, XP000867031 abstract page 76, column 2, paragraph 2 ---	1-63
	--- -/--	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/00800

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NIMNI M E: "Polypeptide growth factors: targeted delivery systems"  BIOMATERIALS, GB, ELSEVIER SCIENCE PUBLISHERS BV., BARKING,  vol. 18, no. 18, page 1201-1225  XP004086390  ISSN: 0142-9612  page 1205, column 1, paragraph 3  page 1208, column 1, paragraph 3; figure 8  ---</p>	1-63
X	<p>HUBELL J. A.: "Bioactive biomaterials"  CURRENT OPINION IN BIOTECHNOLOGY,  vol. 10, April 1999 (1999-04), pages  123-129, XP000866483  page 125, column 2, paragraph 1  ---</p>	1-63
Y	<p>FOWLKES J L ET AL: "Characterization of glycosaminoglycan-binding domains present in insulin-like growth factor-binding protein-3."  JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 JUN 21) 271 (25) 14676-9., XP000867125  abstract  ---</p>	1-63
P,Y	<p>CAMPBELL P G ET AL: "Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin."  JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 OCT 15) 274 (42) 30215-21., XP000913749  abstract  -----</p>	1-63

# INTERNATIONAL SEARCH REPORT

I national application No.  
PCT/IB 99/00800

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 20,21, 25, 26 complete, 1-19, 27, 28 partially

Matrix comprising:

- 1) a substrate capable of providing attachment of a heparin-binding peptide
- 2) a peptide high affinity heparin binding domain
- 3) heparin or heparin-like polymer
- 4) growth factor or peptide fragment with low affinity for heparin.

2. Claims: 45 complete, 29-44, 52- 63 partially

Matrix comprising:

- 1) fibrin as a substrate capable of providing attachment of a heparin or heparin like polymer
- 2) heparin
- 3) growth factor or peptide fragment with low affinity for heparin.

Article for treatment of dermal wounds comprising said matrix and method for controlled release of growth factor placing said matrix on a wound.

3. Claims: 46 complete, 29-44, 52- 63 partially

Matrix comprising:

- 1) collagen as a substrate capable of providing attachment of a heparin or heparin like polymer
- 2) heparin
- 3) growth factor or peptide fragment with low affinity for heparin.

Article for treatment of dermal wounds comprising said matrix and method for controlled release of growth factor placing said matrix on a wound.

4. Claims: 47 complete, 29-44, 52-63 partially

Matrix comprising:

- 1) hyaluronic acid or derivative as a substrate capable of providing attachment of a heparin or heparin like polymer
- 2) heparin
- 3) growth factor or peptide fragment with low affinity for heparin.

Article for treatment of dermal wounds comprising said matrix and method for controlled release of growth factor placing said matrix on a wound.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. Claims: 48 complete, 29-44, 52- 63 partially

Matrix comprising:

- 1) synthetic polymer hydrogel as a substrate capable of providing attachment of a heparin or heparin like polymer
- 2) heparin
- 3) growth factor or peptide fragment with low affinity for heparin.

Article for treatment of dermal wounds comprising said matrix and method for controlled release of growth factor placing said matrix on a wound.

6. Claims: 49-51 complete 29-44, 52- 63 partially

Matrix comprising:

- 1) a substrate capable of providing attachment of a heparin or heparin-like polymer
- 2) heparin-like polymer
- 3) growth factor or peptide fragment with low affinity for heparin.

Article for treatment of dermal wounds comprising said matrix and method for controlled release of growth factor placing said matrix on a wound.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 99/00800

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9616983	A	06-06-1996	US 5654267 A	05-08-1997
			AU 4412396 A	19-06-1996
			CA 2206175 A	06-06-1996
			EP 0797584 A	01-10-1997
			JP 10509980 T	29-09-1998
			US 5830504 A	03-11-1998
WO 9954359	A	28-10-1999	AU 3398999 A	08-11-1999
WO 9921588	A	06-05-1999	AU 1039199 A	17-05-1999
WO 9209301	A	11-06-1992	AU 667188 B	14-03-1996
			AU 9109391 A	25-06-1992
			CA 2097063 A	28-05-1992
			EP 0564502 A	13-10-1993
			JP 6506191 T	14-07-1994
			US 6054122 A	25-04-2000
EP 0838219	A	29-04-1998	AU 3990797 A	23-04-1998
			CA 2217134 A	09-04-1998
			JP 10167987 A	23-06-1998
			NZ 328898 A	25-02-1999
			US 5922356 A	13-07-1999
EP 0732105	A	18-09-1996	US 5693341 A	02-12-1997
			CA 2165727 A	17-09-1996
			JP 8253429 A	01-10-1996
DE 19841698	A	16-03-2000	WO 0015248 A	23-03-2000
WO 0013710	A	16-03-2000	NONE	